Apoptosis in Giant Cell Tumors of Bone

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Summary: Although giant cell tumor of bone (GCT) is characterized by the extensive multinucleated giant cells among mononuclear stromal cells, proliferation of these cells and multinucleation are not without limit in certain cases. Few studies on oncogenesis of GCT have focused on the negative growth control, including growth arrest and apoptosis. The purpose of this study was to investigate the mechanism of cell death in multinucleated giant cells and stromal cells of GCT. In this study, we have demonstrated that GCT cells can undergo apoptosis. The cells in surgical specimen were positively stained in situ nick end labeling methods, and electron micrographs showed the morphological changes associated with apoptosis in some of stromal cells and multinucleated giant cells. A candidate responsible for this apoptosis was then examined using cultured GCT cells. We focused on Fas that is a major trigger of apoptosis. Cultured GCT cells expressed detectable amount of Fas on their surface. Although GCT cells did a little undergo apoptosis following treatment with anti-Fas alone, combination treatment with cyclohexamide led to an increase in apoptosis of the GCT cells. These data suggested that the sensitizing activity of cyclohexamide on anti-Fas mediated cytotoxicity could happen in vitro.

Key words apoptosis, giant cell tumor, Fas, soluble Fas, protein synthesis inhibitor

INTRODUCTION

There are still many unknown issues in giant cell tumor of bone (GCT). The extent of bone destruction is variable case by case; some GCTs are, like aneurysmal bone cysts or malignant bone tumors, in a progressing state invading into the surrounding soft parts, and some are, in a steady state achieved by a balance between bone destruction and formation [1]. GCT is histologically composed of multinucleated giant cells, mononuclear stromal cells of histiocytic and fibroblastic types, and other cells, and their composition has been variable, case by case [2]. The multinucleated giant cells in GCT are characterized by their extraordinarily large number of nuclei compared to those of osteoclasts or giant cells responsive to foreign bodies. However, this multinucleation is not without limit; in certain cases, multinucleated cells are small and have only several nuclei [3]. A careful histological examination of GCT has demonstrated that there are some small multinucleated giant cells and mononuclear stromal cells undergoing degeneration [2]. They have eosinophilic and vacuolated cytoplasm and picnotic nuclei. Focal necrotic areas are scattered in some cases [4,3].

Studies on GCT have mainly been performed in terms of the mechanism by which the tumor grows and destroys bone tissue [5,6]. However, the mechanism by which the tumor growth is repressed remains unknown [2-4], and research is necessary to understand the pathophysiology of GCT. It is generally known that neoplastic transformation of cells leads to an increased tendency to apoptosis. We have thus hypothesized that apoptosis of multinucleated giant cells and stromal cells might increase in GCT to regulate the tumor regression. In this study, we examined the possibility that apoptosis occurred in GCT, using morphological and biochemical techniques. Fas is a 45-kD cell surface glycoprotein that transduces cellular death signals for apoptosis in certain neoplastic cells [7]. We also studied the expression of Fas and the susceptibility/resistance of
GCT cells to Fas-mediated apoptosis.

MATERIALS AND METHODS

Surgical specimen

Specimens were obtained from four patients with GCT. They were named as GM, GT, GY, and GI: GM of a 25-year-old male and GI of a 23-year-old male were found in the distal femur. The case of GT originated from the proximal humerus and extended to the surrounding soft parts of a 27-year-old male. The GY-case was in the distal femoral metaphysis and epiphysis of a 62-year-old female. The tumor destroyed the cortex and extended to the surrounding muscles. These samples were prepared for investigations such as morphology and cell culture.

In situ nick end labeling methods

The surgical specimen were fixed in 4% neutral buffered formaldehyde for 18 hrs and embedded in paraffin. The sections were deparaffined through xylene and graded alcohol, prior staining. Apoptosis was detected by the modified terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labeling (TUNEL) method according to Gavrieli et al. [8] In brief, after proteinase K digestion (20 µg/ml) and removal of endogenous peroxidase with 2% H2O2, the sections were incubated at 37 ºC for 1 hr in a solution containing TdT and digoxigenin-labeled dUTP and deoxyadenosine triphosphate (dATP). The sections were then treated with the peroxidase-labeled anti-digoxigenin antibody solution for 30 min. The reaction products were developed with 3,3’-diaminobenzidine tetrahydrochloride and counterstained with methyl green.

Cell culture

Cell culture systems were used to investigate the mechanism of apoptosis in GCT. Four cases of GCT transferred into cell culture systems were examined in this study. The cells were grown in culture medium consisting of Dulbecco’s MEM (Nissui Seiyaku, Japan) supplemented with 10% fetal bovine serum (Bioserum, Victoria, Australia), 100 U/ml penicillin, 100 µg/ml streptomycin (GIBCO/Life Technologies Inc, Gettysburg, MD), and 12 mM sodium bicarbonate in a humidified atmosphere of 5% CO2/95% air at 37 ºC.

Flow cytometric analysis

Flow cytometric analyses were performed to determine semiquantitatively Fas expression on cultured GCT cells. Cells (4.0×10⁵) were washed once in washing-buffer (10 mM PBS, pH 7.4/0.2% bovine serum albumin/0.1% NaNO₃) and reacted with 10 µl of anti-Fas antibody (IgG₁, clone UB2) on ice for 1 hr. Cells were then washed twice in washing-buffer, incubated with 4 µl of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin on ice for 30 min in a dark place, washed twice in washing-buffer, fixed in 4% paraformaldehyde on ice for 10 min, washed in washing-buffer, and then analyzed using a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA). A negative control was prepared, using normal mouse IgG₁ as the primary antibody.

Immunofluorescence

The Fas protein was detected using the anti-Fas antibody (IgG₁, clone UB2, MBL, Nagoya, Japan) as the primary antibody. The cells grown on a Lab-Tek Tissue Culture Chamber Slide were washed with PBS three times, fixed in 4% paraformaldehyde containing 8% sucrose for 1 hr at room temperature, washed with PBS, frozen immediately by spraying freon gas on the back of the slide, thawed, and used. The anti-Fas antibody was used at a concentration of 10 µg/ml. For negative control, normal mouse IgG₁ was used as the primary antibody.

RT-PCR

A GeneAmp RNA PCR kit (Perkin Elmer Cetus, Norwalk, Conn., USA), which employed the reverse transcriptase-polymerase chain reaction (RT-PCR) method, was used to isolate the Fas and soluble Fas genes from the cells. The RNA was extracted from the cells using ISOGEN-LS (WAKO, Osaka, Japan). For first-strand cdNA synthesis, 100 ng of total RNA was incubated at 37 ºC for 1 hr in 20 ml reverse transcriptase buffer (50 mM Tris-HCl, pH 8.3/75 mM KCl/3 mM MgCl₂/0.5 mM dodecyri-

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HC1, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 0.2 mM deoxyribonucleoside triphosphate, 2 mM of each primer, and 25 U/ml AmpliTaq DNA Polymerase (Perkin Elmer, Branchburg, NJ), using the PCR method. Each cycle included denaturation at 94 °C for 30 sec, annealing at 65 °C for 1 min, and primer extension at 72 °C for 1 min 30 sec. The following primers were designed to detect a main "short" Fas transcript, which is generated via alternative splicing and encoding for a soluble Fas molecule lacking the transmembrane domain [9]: 5'-GAAGGACATGGCTTAGAAGTGG-3' (519-540) and 5'-GGTTGGAGATTCATGAGAACC-3' (complementary to 816 to 836). The size of PCR products were expected to be 318 bp for normal Fas cDNA and 255 bp for a Fas cDNA variant encoding a soluble Fas molecule. Beta-actin message was also amplified under the same conditions using the specific primers [10]: 5'ACCCGTGAAGTACCCCAT-3' (237 to 253) and 5'-TAGAAGCATTTGCGGTG-3' (complementary to 1152 to 1168). The products were electrophoresed in a 2% NuSieve agarose gel containing 0.5% ethidium bromide, visualized by using an UV illuminator, and photographed.

Assessment of anti-Fas effects on cell viability

GCT cells (1.0×10^4 cells/well) was seeded onto a 96-well plate (Falcon) and cultured for 24 hrs. Then the culture medium was exchanged for 100 µl of culture medium containing 200 ng/ml anti-Fas (IgM, clone CH-11, MBL, Nagoya, Japan) with or without 1 to 10 µg/ml cyclohexamide (CHX), and the cells were cultured for 12 hrs. The MT3T (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide) assay [11] was performed to examine the effects of anti-Fas on cell viability.

Morphometry

GCT cells were cultured in medium containing 200 ng/ml anti-Fas (IgM, clone CH-11) with 5 µg/ml CHX, and morphological changes were examined using microscopy and electron microscopy. The ultrastructural studies were performed after the 6 hrs treatment. A certain number of cells became shrunk and detached by this procedure, and ultrastructural examinations were performed only on cells remaining attached.

Cell cycle analysis

Cells were treated with 200 ng/ml anti-Fas (IgM, clone CH-11) with 5 µg/ml CHX for 18 hrs. Treated and control untreated cells were released from the substratum using Passage-ease and permeabilized at 70% ethanol. After centrifugation, cell pellets were resuspended in 1×10^6/ml of PBS, treated with RNase A (1.0 µg/ml) at 37 °C for 30 min, and stained with propidium iodide (PI, 50 µg/ml) for 30 min. The cells were analyzed using a FACScan and a cell cycle analysis doublet discrimination protocol.

Statistics

The significance of any difference was analyzed using Student’s t test (two-tailed).

RESULTS

In situ nick end labeling method

A large number of the nuclei of each of multinucleated giant cells and of stromal cells were positively stained (Fig. 1). There was no difference in staining between histiocytic and fibroblastic stromal cells. Positive granules were localized to the nuclei of both stromal and multinucleated cells and not found in the cytoplasm. As a negative control, phosphate-buffered saline was substituted for TdT containing digoxigenin-labeled dUTP and dATP, which resulted in no staining.

Histological examination of surgical specimen

Cells with morphological changes associated with apoptosis were observed. The giant cell was

Fig. 1. Identification of apoptotic cells in GCT by in situ nick end labeling method. A large number of nuclei of multinucleated giant cells and stromal cells were positively stained. As a negative control, phosphate-buffered saline was substituted for TdT containing digoxigenin-labeled dUTP and dATP, which resulted in no staining. Original magnification, ×200
Fig. 2. Light microscopy on surgical samples.
a, b: An apoptotic multinucleated giant cell with many dense materials as large as nuclei, suggesting nuclear fragmentation. The cytoplasm is smaller than normal multinucleated giant cells. Cytoplasmic membrane was intact.
c: Apoptotic stromal cells having smaller cytoplasms and darker nuclei. Original magnification, ×200

Fig. 3. Flow cytometric analysis of Fas protein expression on cultured GCT cells demonstrating detectable Fas levels on their surface.
smaller and darker than normal multinucleated giant cells, having many vacuoles and large dense materials as large as nucleus, suggestive of nuclear fragmentation. Cytoplasmic membrane was intact. These figures were very mimic to them reported by Blanco et al. [12] and Itoh et al. [7]. The stromal cell that was smaller and darker than normal stromal cells were also observed (Fig. 2).

Flow cytometric analysis of Fas

From those findings obtained using surgical samples, it was suggested that apoptosis could happen in GCT. A candidate responsible for apoptosis was then examined using cultured stromal cells. We focused on the Fas protein, which is a cell surface glycoprotein that transduces cellular death signals for apoptosis in normal and certain neoplastic cells. Flow cytometric analysis was performed to determine semiquantitatively the Fas expression. Flow cytometric analysis of the cultured GCT cells composed of mononuclear cells alone showed detectable levels of Fas antigen in all four samples (Fig. 3).

Immunohistochemistry for Fas

The Fas protein was detected immunohistochemically, using the anti-Fas antibody (IgG, clone UB2), as the primary antibody. Fas immunoreactivity was demonstrated both on the cell surface and in the cytoplasm (Fig. 4). The cells reacted with normal mouse IgG, as the primary antibody showed no reaction.

Detection of Fas and soluble Fas messengers

RT-PCR methods demonstrated a 318 bp band of DNA fragment, which corresponds to the expected size of normal Fas cDNA. In addition to this band, a 255 bp band of DNA fragment, which is equivalent to a Fas cDNA variant encoding a soluble Fas molecule, was identified. The amount of variant transcript was smaller than that of normal Fas transcript (Fig. 5). PCR reactions using beta-actin specific primers demonstrated a distinct single 931 bp band in all samples (Fig. 5).

Assessment of anti-Fas effects on cell viability

Since Fas is a trigger of apoptosis, antibody to Fas seemed to induce apoptosis in GCT cells. However, the treatment of cultured GCT cells with anti-Fas alone resulted in a little effect on cell inhibition. The simultaneous administration of CHX increased the sensitivity of all cell strains to apoptosis induced by antibody to Fas (Fig. 6). CHX alone gave very low...
Fig. 6. Assessment of anti-Fas effects on cell viability showing that treatment of cultured GCT cells with anti-Fas alone resulted in a little effect on cell proliferation. However, cyclohexamide (CHX) increased the sensitivity of GCT cells to Fas-induced apoptosis. CHX alone gave very low levels of toxicity in GM and GI cases. White bars: Fas (-); black bars: Fas (+); p<0.001 versus control (the leftest white bar).

Fig. 7. The morphological appearance of cultured GCT cells treated by anti-Fas and cyclohexamide (CHX) for 12 hrs showing detached rounded-up and floating cells in the culture medium. Left: control; Right: cells treated by anti-Fas and CHX. Original magnification, ×100.
Fig. 8. Ultrastructural examination of cultured GCT cells. 

a: Untreated cells. Original magnification, ×1,500
b: Cells treated by anti-Fas and cyclohexamide for 6 hrs became shrunken and dark. The cytoplasmic organelles were sparse, and there were many vacuoles. Nuclear chromatin were accumulated and condensed, and large nucleoli were evident. Original magnification, ×2,000
c: A cell in the late phase of apoptosis showing more prominent vacuoles, nuclear fragmentation, and cytoplasmic and nuclear blebs. Original magnification, ×3,000

levels of toxicity in GM and GI cases.

Morphometric evaluation

An examination using phase-contrast microscopy revealed that the cells treated with anti-Fas and CHX became detached, round, and floated in the culture medium within 12 hrs after initiation of treatment (Fig. 7). An ultrastructural examination of cells treated by anti-Fas and CHX for 6 hrs revealed shrunken cells with paucity of cytoplasmic organelles, vacuoles, nuclear shrinking accompanied by margination and condensation of the chromatin, and large round

Fig. 9. Assessment of anti-Fas effects on the cell cycle using flow cytometric analysis demonstrating that PI staining revealed a progressive decrease in the diploid peak and an increase in the number of hypoploid cells.
nucleoli (Fig. 8). Those morphological features were consistent with apoptosis.

Cell cycle analysis

PI staining revealed a progressive decrease in the diploid peak and an increase in the number of hypoploid cells. This was a pattern of DNA fragmentation suggesting induction of apoptosis in the treated cells (Fig. 9).

DISCUSSION

Apoptosis is the normal mechanism of cell deletion in embryonic development, metamorphosis, hormone-dependent atrophy of tissues, and in lymphocyte maturation in the thymus [13,14]. Its execution plays a major role in the control of shape and size in normal and abnormal processes [15]. Apoptosis exerts a homeostatic function in relation to tissue dynamics, as the steady state of continuously renewing tissues is achieved by a balance between cell replication and cell death. Apoptosis has also been demonstrated in cells in response to glucocorticoids [16,17], to various chemotherapeutic agents [18,19], and in response to radiation [20,21]. Spontaneous apoptosis is a feature of some tumors and is implicated in the mechanism for regulating tumor growth and maintaining homeostasis [22,23]. Apoptotic cells are physiologically and morphologically different from necrotic cells. In the early stages of apoptosis, the cell rounds up, severs connections with neighboring cells, and loses its microvilli. The cytoplasm and nuclei characteristically form dense blebs. Chromatin condensation in the apoptotic nucleus is associated with endogenous endonuclease activity [16,24]. The apoptotic fragments are engulfed, either by neighboring cells, or macrophages, within which they undergo hydrolytic phagocytic degradation [22].

In this study, we have demonstrated clearly for the first time that GCT cells can undergo apoptosis, characterized by both ultrastructural changes and DNA fragmentation. Apoptosis, a negative control mechanism, seems to be present in GCT to regulate tumor growth. As a candidate for the factor that triggers apoptosis in GCT, we have demonstrated the Fas antigen in stromal cells. The presence of Fas on osteoclastic giant cells in GCT has been previously suggested [25]. The Fas antigen is an apoptosis-related protein generally located on the cell surface, which encodes a transmembrane signaling domain. The Fas antigen belongs to the same family of proteins as tumor necrosis factor (TNF) receptor, nerve growth factor, CD40, CD27, and CD30 [7,26]. The 80-amino-acid cytotoxicity-signaling domain (death domain) within a TNF-receptor contains a region of 65 amino acids that shares a 28% homology with a region within the intracellular domain of the Fas antigen [27]. Although this homology is not extensive, the Fas antigen can signal apoptosis similar to that signaled by the TNF-receptor [7]. Multinucleated giant cells in GCT were reported to be positive for TNF, whereas stromal cells were negative for TNF [28,6]. Neale [25] demonstrated absence of TNF receptor on multinucleated giant cells. Therefore, a paracrine mechanism of TNF against stromal cells might be present in multinucleated giant. It is also possible that Fas-mediated apoptosis in GCT is due to the action of cytotoxic T cells (CTLs). It has been demonstrated that CTLs express the Fas ligand on their surface and that the interaction of the Fas ligand with Fas on target cells induces apoptosis [29].

Despite the presence of detectable Fas, GCT cells did a little undergo apoptosis following treatment with the anti-Fas antibody in vitro. Two different potential mechanisms of resistance are considered. The first one occurs at a pre-receptorial level and involves the production of a soluble, secreted and potentially neutralizing form of Fas [9]. In the present study, a soluble form of Fas was demonstrated in cultured GCT cells. However, the amount of variant transcript was very small compared to normal Fas transcript.

The second mechanism of resistance, which is post-receptorial, was overcome by CHX. It was reported that CHX could increase the sensitivity of many cell types to apoptosis induced by both TNFα and anti-Fas [30,31]. To evaluate whether the increase in Fas expression on the GCT cell surface was the mechanism by which CHX rendered GCT cells sensitive to anti-Fas, we have analyzed the Fas expression after CHX pretreatment, and found this was not the mechanism (data not shown). The simplest explanation for this sensitizing effect is interpreted as the consequence of the inhibition of the synthesis of short-lived proteins having the ability to protect the target cell from TNFα or Fas-induced apoptosis. However, it has been reported that this sensitizing effect of CHX can take place even at concentrations that are far lower than that required for efficient blocking of protein synthesis [32].

An alternative explanation is that CHX can act to modulate cellular signaling properties. It has been
known that the induction of apoptosis involves activation of a signaling system. It is interesting that CHX is able to activate in certain cells a subfamily of c-Jun kinase (Stress-Activated Protein Kinase, SAPK/JNK) related to MAP-kinases and also activated by other apoptotic stimuli, including TNFα and Fas ligand [33,34]. However, many elements of this signaling system still remain to be solved.

In conclusions, we demonstrated for the first time that apoptosis is a genuine feature of GCT. We have also characterized the effects of Fas engagement in GCT. Although the Fas antigen was detected on their cell surface, there was resistance to anti-Fas in vitro. A soluble, secreted and potentially neutralizing form of Fas was detected. However, since its amount was small compared to normal Fas, its role remained uncertain. The resistance to anti-Fas was overcome by CHX. The exact mechanism may be clarified in terms of intracellular signaling pathways. Cytokines including IL-6 and INFγ may be a factor exerting the role of CHX in vivo. We believe our findings should facilitate understanding of the mechanisms involved in the triggering of disease-regression in GCT.

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